

# Requirements for the destruction of human Aurora-A

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## Summary

The mitotic kinase Aurora A (Aur-A) is overexpressed in a high proportion of human tumors, often in the absence of gene amplification. In somatic cells, Aur-A protein levels fall following mitosis or upon overexpression of Cdh1, an activator of the ubiquitin ligase APC/C. Thus, mutations that reduce or block the rate of Aur-A destruction might also be expected to contribute to its oncogenic potential. Previous work had defined two short sequences of *Xenopus* Aur-A that are required for its Cdh1-inducible destruction in extracts of *Xenopus* eggs, an N-terminal A box and a C-terminal D box, and a serine residue within the A box whose phosphorylation might inhibit destruction. Here, we show that these same sequences are required for the destruction of human Aur-A during mitotic exit and G1 in the somatic cell cycle. Expression of a dominant negative Cdh1 protein leads to accumulation of Aur-A, further indicating that the Cdh1-activated form of the APC/C is

responsible for destruction of Aur-A during the somatic cell cycle in vivo. During the course of this work, we found some previously unsuspected problems in commonly used in vitro destruction assays, which can result in misleading results. Potentially confounding factors include: (i) the presence of D-box- and A-box-dependent destruction-promoting activities in the reticulocyte in vitro translation mix that is used to produce radiolabeled substrates for destruction assays; and (ii) the ability of green-fluorescent-protein tags to reduce the destruction rate of Aur-A substantially. These findings have direct relevance for studies of Aur-A destruction itself, and for broader approaches that use in vitro translation products in screens for additional APC/C targets.

Key words: Aurora kinases, Mitosis, *Xenopus*, Cdh1, A box, Ubiquitin-dependent proteolysis, Cell cycle

## Introduction

The serine/threonine kinase Aurora-A (Aur-A) plays several crucial roles during cell division, including mitotic entry (Andresson and Ruderman, 1998; Mendez et al., 2000; Hirota et al., 2003), the formation and maintenance of bipolar spindles (Glover et al., 1995; Hannak et al., 2001), chromosome segregation, and cytokinesis (Marumoto et al., 2003). Forced overexpression of Aur-A in vertebrate somatic cells interferes with chromosome segregation and cytokinesis (Littlepage et al., 2002; Meraldi et al., 2002; Anand et al., 2003; Kunitoku et al., 2003), leading, in certain cases, to oncogenic transformation and tumor formation in nude mice (Bischoff et al., 1998; Zhou et al., 1998; Littlepage et al., 2002). Aur-A protein is overexpressed in a remarkably high proportion of human cancers (Sen et al., 1997; Bischoff et al., 1998; Zhou et al., 1998; Takahashi et al., 2000; Miyoshi et al., 2001; Tanaka et al., 2002; Li et al., 2003) and has therefore attracted considerable interest as a potential drug target. Notably, Aur-A protein overexpression in cancer does not always correlate with Aur-A gene amplification (Sakakura et al., 2001; Miyoshi et al., 2001); mutations that affect Aur-A synthesis or destruction might also lead to abnormally high levels of Aur-A protein.

In somatic cells, both the protein levels and the kinase activity of Aur-A peak during mitosis, and then fall (Bischoff et al., 1998; Lindon and Pines, 2004). The addition of proteasome inhibitors to cycling cells leads to the accumulation

of ubiquitinated forms of Aur-A (Honda et al., 2000; Walter et al., 2000), consistent with regulated destruction of Aur-A through the ubiquitin-proteasome pathway. The destruction of several mitotic regulators is mediated by the multisubunit ubiquitin ligase APC/C (anaphase-promoting complex/cyclosome), whose substrates invariably contain a short destruction-box (D-box) sequence. Aur-A contains three D-box motifs. Extracts of G1-phase *Xenopus* somatic cells can catalyze the destruction of recombinant Aur-A protein, dependent upon the most C-terminal D-box sequence (Arlot-Bonnemains et al., 2001). Two alternative activators, Cdc20 and Cdh1, determine the timing and substrate specificity of APC/C activity during mitotic exit (for a review, see Peters, 2002). Forced overexpression of Cdh1 in somatic cells reduces the level of co-expressed Aur-A protein, suggesting that Aur-A is destroyed by the APC/C-Cdh1 pathway (Taguchi et al., 2002). The most detailed information about Aur-A destruction so far has been obtained using extracts of *Xenopus* eggs, in which Aur-A is not normally destroyed (Littlepage and Ruderman, 2002; Castro et al., 2002). This statement might seem paradoxical, but such studies are in fact possible because *Xenopus* eggs lack Cdh1, and addition of Cdh1 permits the destruction of Aur-A. Briefly, eggs are naturally arrested at metaphase of meiosis II (MII), but fertilization induces the activation of APC/C-Cdc20 and exit from MII into the first embryonic cell cycle. This cell-cycle transition can be recapitulated in egg extracts by adding calcium to mimic

fertilization. Unlike somatic cells, eggs lack Cdh1 (Lorca et al., 1998; Zhou et al., 2002); addition of recombinant Cdh1 protein to egg extracts permits the formation of the Cdh1-activated form of APC/C during MII exit (Pfleger and Kirschner, 2000; Pfleger et al., 2001). Under these conditions, Aur-A is destroyed rapidly (Littlepage and Ruderman, 2002; Castro et al., 2002). Addition of radiolabeled in vitro translation (IVT) products encoded by wild-type or mutant *Xenopus* Aur-A to egg extract supplemented with Cdh1 allowed further mapping of the sequences required for Aur-A proteolysis. These include a D box in the C-terminal catalytic domain, a short region in the N-terminal non-catalytic domain termed the A box (Littlepage and Ruderman, 2002; Castro et al., 2002) and serine 53 within the A box, whose phosphorylation appears to regulate the destruction of Aur-A negatively (Littlepage and Ruderman, 2002). Surprisingly, the N-terminal KEN sequence, previously identified as a Cdh1 recognition signal in other proteins, is not required for the destruction of *Xenopus* Aur-A (Arlot-Bonnemains et al., 2001; Littlepage and Ruderman, 2002).

Several obvious questions remain. Aside from the D box, nothing is known about the sequence requirements for destruction of Aur-A during the somatic cell cycle. Although overexpression of Cdh1 led to reduced levels of co-expressed Aur-A (Taguchi et al., 2002), it is not known whether Cdh1 is required to regulate Aur-A levels in cells. Despite the expectation that the rate of Aur-A destruction increases during late mitotic exit, there has so far been no formal demonstration of this. For example, mitotic extracts were not directly compared with the G1-phase cell extracts that were found to destroy *Xenopus* Aur-A IVT product (Arlot-Bonnemains et al., 2001; Klotzbucher et al., 2002), and conclusions drawn from experiments using proteasome inhibitors to address the same question (Honda et al., 2000) are complicated by the fact that proteasome function is required at several points during mitotic exit. Here, we report that the destruction of human Aur-A during mitotic exit and G1 in somatic cells requires the same destruction signals previously defined for *Xenopus* Aur-A, including the A box.

The characterization of APC/C substrates is frequently conducted by incubation of extracts from a particular cell cycle stage with radiolabeled IVT products made in rabbit reticulocyte lysate (Luca and Ruderman, 1989; Brandeis and Hunt, 1996; McGarry and Kirschner, 1998; Bastians et al., 1999; Pfleger and Kirschner, 2000). Radiolabeled IVT products are added to cell extracts and their proteolysis monitored by autoradiography of samples taken at subsequent time points. Furthermore, the use of IVT products encoded by pools of cDNAs has been widely used in screens to search for new APC/C substrates (McGarry and Kirschner, 1998; Zou et al., 1999; Funabiki and Murray, 2000; Ayad et al., 2003). During the course of this work, we uncovered two aspects of this commonly used assay that can result in potentially misleading results. (i) IVT human Aur-A, but not *Xenopus* Aur-A, is subject to A-box- and D-box-dependent proteolysis in reticulocyte lysate in the absence of other cell extracts. (ii) A green fluorescent protein (GFP) tag can reduce the efficiency of Aur-A destruction. These results have implications for the design of screening strategies to discover new human APC/C substrates, and for the analysis of the destruction kinetics of GFP-tagged Aur-A, and perhaps other APC/C substrates, in vivo.

## Materials and Methods

### Plasmids, proteins and antibodies

Full-length human Aur-A was cloned by polymerase chain reaction (PCR) from human thymus (QUICK-Clone cDNA, BD Biosciences), using the Advantage cDNA cloning kit (Clontech). PCR products were cloned into the *Bam*HI/*Xho*I sites of pCS2+ (obtained from M. Kirschner, Harvard Medical School, Boston, MA). Truncation mutants were produced by PCR (Expand polymerase, Roche) and point mutants were generated using the QuikChange mutagenesis kit (Stratagene). His<sub>6</sub>-tagged Cdh1(1-125) was subcloned into pBABLE-puro (obtained from G. Nolan, Stanford University, CA) by PCR from a template kindly provided by C. Pfleger (Harvard Medical School). All constructs were verified by DNA sequencing (Dana-Farber/Harvard Cancer Center sequencing facility). Primer sequences are available from us on request. Plasmids encoding *Xenopus* Aur-A (Andresson and Ruderman, 1998), human cyclin B1 (Bastians et al., 1999) and human UbcH10 (Bastians et al., 1999) have all been described previously. The following colleagues generously supplied plasmids. Myc tag, Myc-Cdh1, Myc-Cdc20 and His<sub>6</sub>-Cdh1(1-125) were from C. Pfleger and M. Kirschner. Flag-CUL1(1-452) was from Z.-Q. Pan (Mount Sinai School of Medicine, New York, NY). Antibodies used were as follows. Human Aurora-A, IAK-1 (BD Biosciences). Histidine tag, HIS-1 (Sigma). p27, C-19 (Santa Cruz Biotechnology). Flag epitope, M2 (Sigma). Cdc2, PSTAIRE (Santa Cruz).

### Proteins

Coupled IVT and translation were performed from pCS2+ constructs using a rabbit reticulocyte lysate system (TnT SP6, Promega), using 0.2 µg phenol-extracted miniprep DNA template per 10 µl reaction, incubated at 30°C for 90 minutes. [<sup>35</sup>S]-Methionine and [<sup>35</sup>S]-cysteine (NEN/PerkinElmer) were used for radiolabeling IVT proteins. mRNA was prepared using mMessage mMachine (Ambion) according to the manufacturer's instructions, and used to prime translation in rabbit reticulocyte lysate (TnT SP6, Promega) at 20 µg ml<sup>-1</sup>. For protein expression in insect cells, Aur-A variants were subcloned into the *Bam*HI/*Xho*I sites of pFastBac-HTb (Invitrogen) and produced in baculovirus according to the manufacturer's instructions. His<sub>6</sub>-tagged proteins were recovered from infected Sf9 cell cultures using nickel-agarose beads (Qiagen) and eluted into buffer containing 5% glycerol, 5 mM HEPES/KOH, pH 7.7, 50 mM NaCl, 50 µM EDTA, 0.5 mM dithiothreitol and protease inhibitors (Complete EDTA-free, Roche). His<sub>6</sub>-Cdh1 and His<sub>6</sub>-Cdc20 were similarly purified from Sf9 cells infected with baculoviruses generously supplied by C. Pfleger. Human UbcH10 was expressed from pET28 vector (Novagen) in *Escherichia coli* BL21 (Stratagene) and purified as previously described (Bastians et al., 1999).

### *Xenopus* egg-extract destruction assays

*Xenopus* eggs, which are arrested in metaphase of MII, were collected and cytostatic factor (CSF)-arrested extracts were prepared (Murray, 1991). Destruction assays were performed as described (Littlepage and Ruderman, 2002). Briefly, 20 µl thawed CSF extract was supplemented with 0.1 mg ml<sup>-1</sup> cycloheximide incubated on ice for 5 minutes with 2 µg human Cdh1 or Cdc20 purified from Sf9 insect cells (baculoviruses were a gift of C. Pfleger). 1 µl substrate protein ([<sup>35</sup>S]-labeled IVT) or 0.5 µg protein purified from Sf9 cells was added for a further 10 minutes before the addition of 0.4 mM calcium chloride in a total reaction volume of 25 µl. Reactions were shifted to 25°C and 5 µl samples were taken at subsequent times for analysis by polyacrylamide-gel electrophoresis (PAGE) and either autoradiography or western blot.

### Cell culture

HeLa cells (CCL-2, American Type Culture Collection) were grown

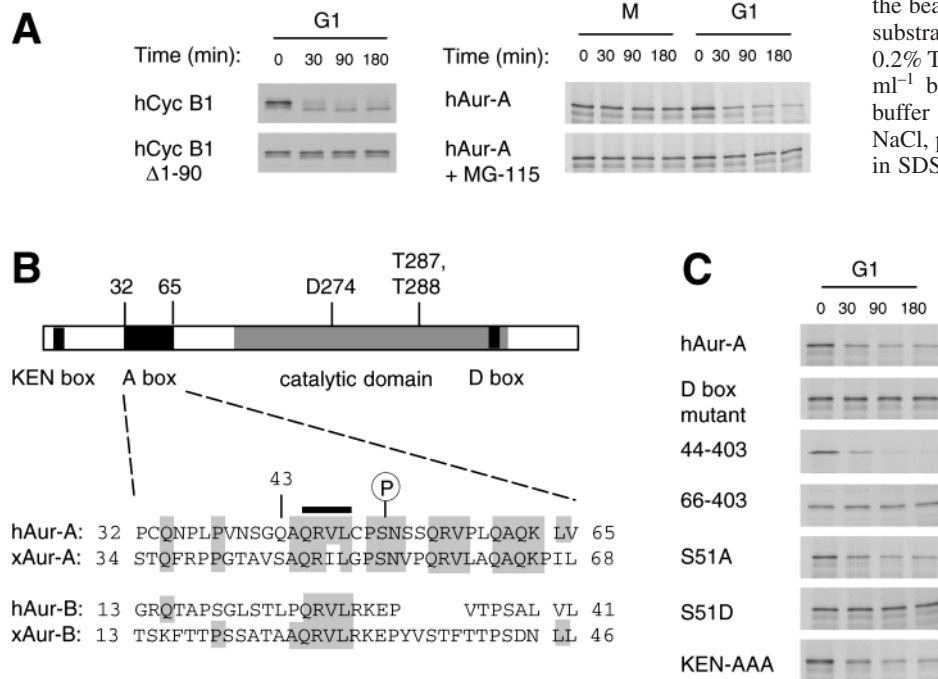
in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with antibiotics and 10% fetal calf serum (Invitrogen). To obtain cells synchronized in M phase, cells were incubated in medium containing 2 mM thymidine (Sigma) for 18 hours, released into fresh medium for 6 hours and then incubated with 0.1  $\mu$ M nocodazole (Sigma) for 16 hours. These cells were released into fresh medium for 4 hours to obtain an early G1-phase population. Transfection of maxiprep CUL1(1-452) DNA into HeLa cells was performed using Lipofectamine reagent (Invitrogen) and OptiMEM serum-free medium (Invitrogen) according to the manufacturer's instructions. Amphitropic retrovirus encoding His<sub>6</sub>-tagged Cdh1 N-terminus and control (empty) retrovirus were constructed by transfection of pBAGE-puro constructs into 293-GPG packaging cells (a kind gift of R. Guwardene and J. Brugge, Harvard Medical School, Boston, MA) using Lipofectamine 2000 reagent (Invitrogen). Viral supernatants were harvested daily from 4 days to 7 days after transfection, pooled and frozen in aliquots at  $-80^{\circ}\text{C}$ . Infection of HeLa cells was performed using 5  $\mu\text{g ml}^{-1}$  polybrene (Sigma). Cell lysates for western blotting were prepared by rinsing in cold PBS and incubation for 10 minutes on ice with lysis buffer containing 50 mM HEPES, pH 7.4, 100 mM KCl, 25 mM NaF, 0.5% NP-40, 1 mM sodium vanadate, 1 mM dithiothreitol and protease inhibitors (Complete EDTA-free, Roche). Lysates were clarified by centrifugation before boiling with sodium-dodecyl-sulfate (SDS) sample buffer.

#### HeLa-cell-extract destruction assays

HeLa-cell extracts were prepared as described previously (Bastians et al., 1999) and stored in aliquots at  $-80^{\circ}\text{C}$ . Thawed 8  $\mu\text{l}$  aliquots on ice were supplemented with ubiquitin (7.5  $\mu\text{M}$ , Sigma) and recombinant UbcH10 (2.5  $\mu\text{M}$ ) to a final volume of 9  $\mu\text{l}$ . 1  $\mu\text{l}$  substrate protein (radiolabeled IVT) or 0.5  $\mu\text{g}$  protein purified from Sf9 cells was added and the extract shifted to  $30^{\circ}\text{C}$ . 2  $\mu\text{l}$  samples were taken at subsequent times for analysis by PAGE and autoradiography or western blot.

#### Cdh1 interaction assays

The method used is derived from Pflieger et al. (Pflieger et al., 2001). Briefly, IVT reactions (rabbit reticulocyte lysate, TnT SP6, Promega) were prepared for Myc tag, Myc-Cdh1 and Myc-Cdc20 without radiolabel; aliquots of each reaction were also incubated in the presence of [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine to allow quantitation of the translation product by SDS-PAGE, autoradiography and phosphorimager analysis (Quantity One software, Bio-Rad). Relative molar amounts of each product were estimated by taking into account the number of methionine and cysteine residues in each, and the volumes of the unlabeled products adjusted accordingly with reticulocyte lysate to give equal product concentrations. 7  $\mu\text{l}$  of each unlabeled product was incubated with 10  $\mu\text{g}$  anti-Myc antibody conjugated to agarose beads (9E10, Santa Cruz Biotechnology) in buffer A (50 mM HEPES, pH 7.7, 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM EDTA). After washes in buffer A, the beads were incubated with 2  $\mu\text{l}$  radiolabeled IVT substrate in binding buffer B (buffer A containing 0.2% Tween-20, 0.1 mg ml<sup>-1</sup> cycloheximide and 1 mg ml<sup>-1</sup> bovine serum albumin). After five washes in buffer B and one in buffer B containing 150 mM NaCl, proteins were eluted from the beads by boiling in SDS sample buffer.



**Fig. 1.** Destruction of human Aur-A protein in G1-phase extracts of HeLa cells requires the D box and the A box. (A) HeLa cells were synchronized by a double thymidine block, released into nocodazole to collect cells in M phase and subsequently released into G1 phase. Extracts were prepared from M-phase and G1-phase populations, and supplemented with ubiquitin and UbcH10. IVT human cyclin B1 or non-degradable cyclin B1 D1-90 (A) or variants of human Aur-A were then added, with the proteasome inhibitor MG-115 where indicated. Samples were taken at the times and processed for SDS-PAGE followed by autoradiography. (B) Schematic of potential destruction signals and catalytic residues in human Aur-A. The sequence alignment compares the A box in human (h) and *Xenopus* (x) Aur-A with the corresponding region in Aur-B. Residues conserved with human Aur-A are shaded. The solid bar indicates the conserved QRxL motif. The circled P indicates the serine phosphorylation site (S51 in human Aur-A). (C) IVT mutants of human Aur-A were assayed for destruction in G1-phase HeLa-cell lysates as described in A.

#### Results

Sequences required for destruction of Aur-A during MII exit in eggs are also used during exit from mitosis in the somatic cell cycle

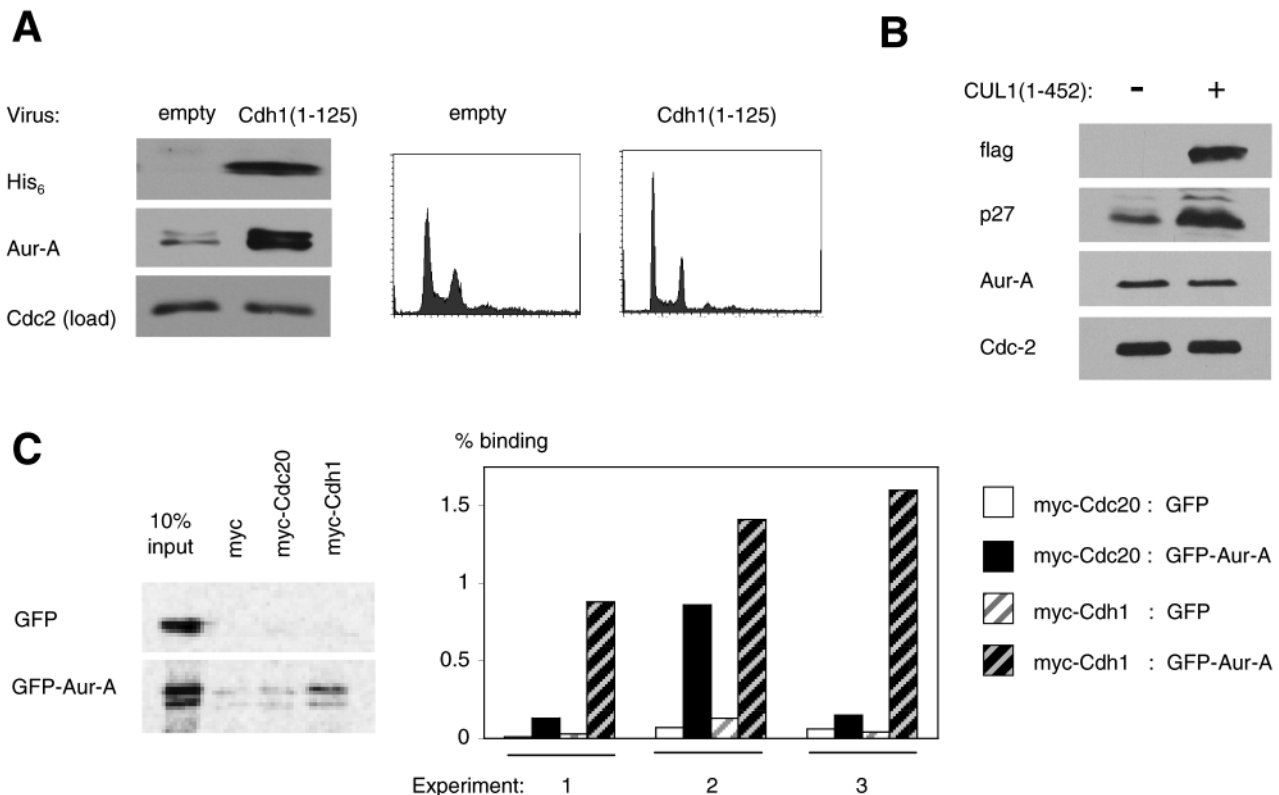
In somatic cells, the destruction of several APC/C targets begins during mitotic exit and continues during G1 of the next cell cycle. We first asked whether the requirements for the destruction of human Aur-A in the somatic cell cycle are the same as those previously defined during the Cdh1-induced destruction of Aur-A during exit from MII in *Xenopus* egg extracts (Littlepage and Ruderman, 2002; Castro et al., 2002). Highly concentrated extracts were prepared from HeLa cells that had been arrested in M phase using nocodazole or from G1-phase cells that had been collected 4 hours after release from nocodazole arrest. [<sup>35</sup>S]-Labeled IVT products were used as substrates in the destruction assays

(Brandeis and Hunt, 1996; Bastians et al., 1999). Human cyclin B1 IVT product was stable in M-phase extracts (data not shown) and degraded by G1-phase cell extracts in a manner dependent on its N-terminal D box (Fig. 1A). Thus, these extracts behaved as previously described. Human Aur-A IVT product was stable in nocodazole-arrested M-phase extracts and destroyed in G1-phase extracts in a proteasome-dependent manner (Fig. 1A). Although not unexpected, this result is in fact the first formal demonstration that the rate of Aur-A proteolysis does indeed increase during the transition from mitosis to G1.

The sequences required for Cdh1-induced destruction of *Xenopus* Aur-A during meiotic exit (Littlepage and Ruderman, 2002; Castro et al., 2002) are well conserved in human Aur-A (Fig. 1B). Using G1 extracts, we next tested the destruction in G1 extracts of human Aur-A IVT products mutated at these sites (Fig. 1C). Mutation of the C-terminal D box (R<sup>371</sup>PML to APMA) blocked destruction. N-terminal truncations revealed that the first 43 residues are not required for destruction (44-403), but removal of the first 65 residues, encompassing the conserved A-box sequence, blocked destruction. In *Xenopus*, S53 within the A box is phosphorylated during M phase; the S53D mutation, which might mimic phosphorylation, blocks

Cdh1-dependent destruction (Littlepage and Ruderman, 2002). Mutation of the equivalent residue in human Aur-A (S51D) also blocked destruction during G1, whereas the mutation S51A did not. Finally, the N-terminal KEN box, which is needed for the destruction of certain other Cdh1 targets (Pfleger and Kirschner, 2000), was not required for Aur-A destruction in G1 (Fig. 1C). Therefore, the same destruction motifs required for the destruction of Aur-A during exit from MII are also used during G1 in the somatic cell cycle.

**Cdh1 is required for destruction of Aur-A in human cells**  
Overexpression of Cdh1 in somatic cells reduces the levels of a co-expressed Aur-A protein (Taguchi et al., 2002). To address whether Cdh1 is required for Aur-A destruction under normal conditions, HeLa cells were infected with retrovirus encoding an N-terminal fragment of Cdh1 protein (residues 1-125), which inhibits the APC/C-dependent destruction of Cdh1 substrates (Pfleger et al., 2001). By 48 hours after infection, the level of endogenous Aur-A was significantly elevated (Fig. 2A). These data suggest that, just as in meiotic exit in eggs, Cdh1 is required for Aur-A destruction during mitotic exit in the somatic cell cycle. Because the cell-cycle profile was not



**Fig. 2.** Cdh1 is required for the destruction of Aur-A in HeLa cells. (A) HeLa cells were infected with retrovirus encoding His<sub>6</sub>-tagged N-terminal dominant negative fragment of Cdh1 [Cdh1(1-125)] or an empty retrovirus control. 24 hours after infection, cells were lysed and processed for western blotting with antibodies against His<sub>6</sub> or Aur-A, as indicated, or for propidium iodide staining and flow cytometry. (B) HeLa cells were transfected with or without pcDNA3-FLAG-CUL1(1-452). 48 hours after transfection, cells were lysed and processed for western blotting with antibodies against FLAG, p27 or Aur-A, as indicated. In both A and B, the Aur-A blot was stripped and re-probed with anti-Cdc2 antibody as a loading control. (C) IVT Myc tag, Myc-Cdh1 or Myc-Cdc20 were incubated with anti-Myc beads and washed extensively. Beads were then incubated with [<sup>35</sup>S]-labeled IVT GFP or GFP/Aur-A variants, as indicated, followed by washes and elution into SDS sample buffer. Samples were analysed by PAGE and autoradiography alongside 10% of the input IVT product, and bands quantified by phosphorimager analysis.

significantly changed by Cdh1(1-125) expression (Fig. 2A), the observed Aur-A accumulation was not an indirect effect of cells arresting in G2/M phase.

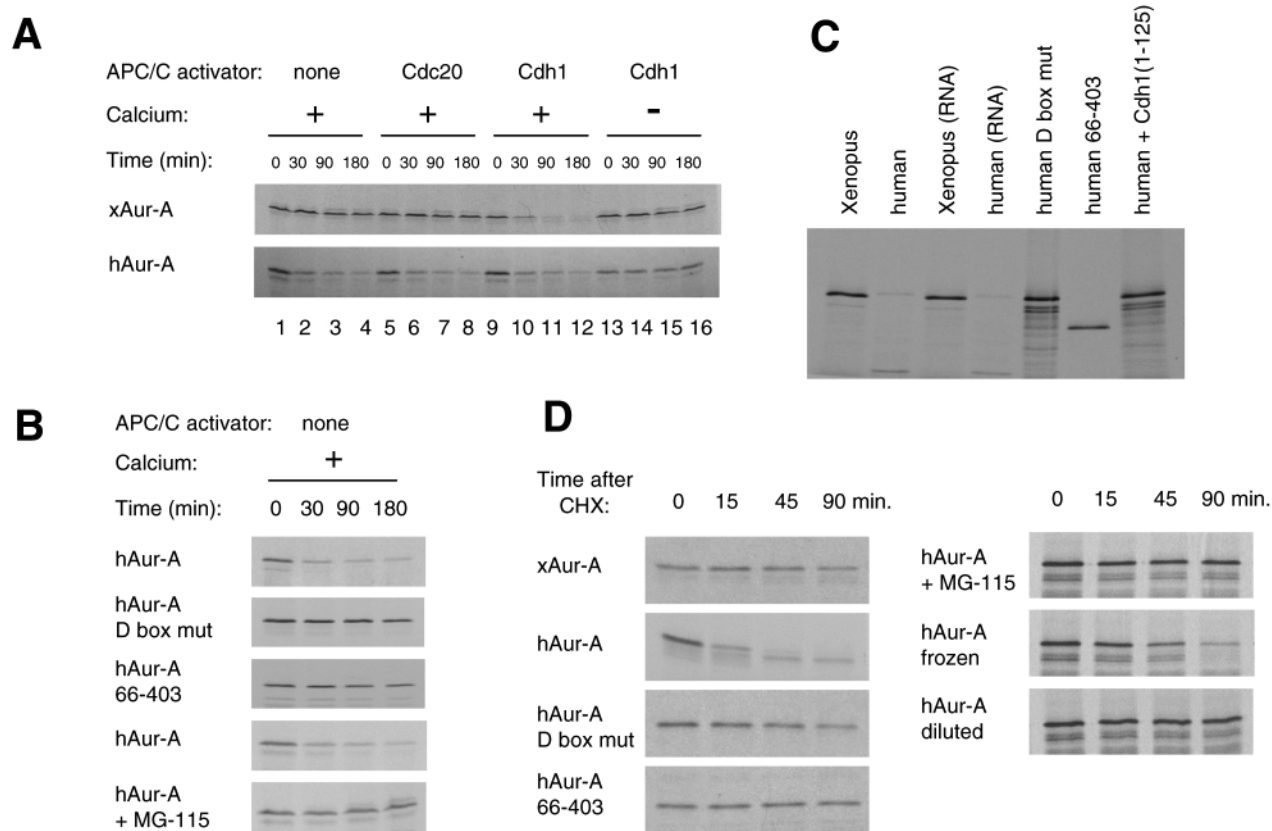
Some proteins, like the Cdc2-activating phosphatase Cdc25A, are targeted for ubiquitination by the APC/C in mitosis and by the SCF (Skp1/cullin/F box) ubiquitin-ligase complex during G1 (Donzelli et al., 2002). SCF subunits have been found at the centrosomes (Freed et al., 1999), where a portion of Aur-A resides. To determine whether Aur-A destruction during G1 might involve an SCF ubiquitin ligase, HeLa cells were transfected with a truncated version of the cullin family protein Cul1. This truncated protein, Cul1(1-452), inhibits the function of all Cul1-containing SCF complexes, including those responsible for Cdc25A destruction (Wu et al., 2000; Donzelli et al., 2002). After 24 hours, levels of p27<sup>Kip1</sup>, a known SCF substrate, were significantly elevated but Aur-A levels were not (Fig. 2B). It therefore seems likely that APC/C-Cdh1 is the sole or predominant ubiquitin ligase mediating Aur-A destruction in vivo.

To investigate possible functional interactions between Cdh1

and Aur-A, unlabeled in vitro translated Myc-tagged Cdh1 bound to anti-Myc beads was used to pull down radiolabeled IVT Aur-A (Pfleger et al., 2001). As described later, it was difficult to obtain high yields of IVT human Aur-A, so we used GFP-tagged Aur-A, which gives much higher IVT yields. Beads loaded with Myc tag alone did not appreciably pull down GFP/Aur-A, whereas Myc-Cdh1 associated with approximately 1% of the input Aur-A (Fig. 2C). This degree of binding is, although low, similar to that previously observed using this assay with other APC/C substrates such as XKid, geminin and securin (Pfleger et al., 2001). Indeed, a recent report suggests that binding of substrates to Cdh1 occurs only transiently (Yamano et al., 2004).

#### Important caveats of using rabbit reticulocyte IVT products as substrates in destruction assays

The results described above demonstrate that the broad requirements for human Aur-A destruction during exit from mitosis in somatic cells are identical to those for *Xenopus* Aur-A during meiotic exit in eggs. We next planned a more detailed



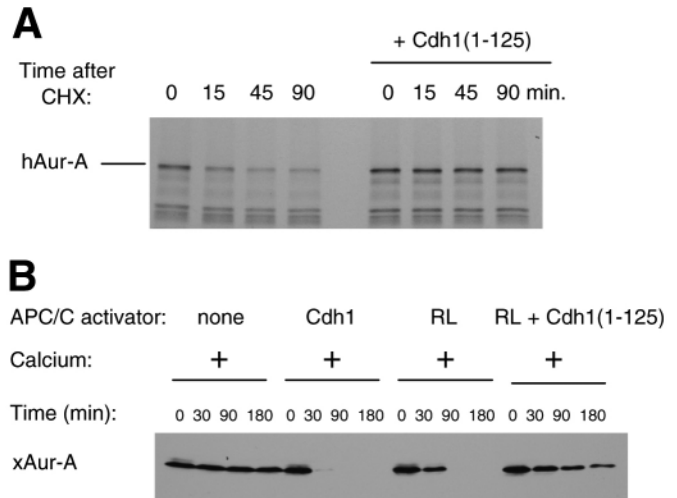
**Fig. 3.** Human Aur-A IVT product is destroyed in egg extract without additional Cdh1. (A,B) *Xenopus* egg CSF extract was incubated for 5 minutes with the APC/C activators Cdc20 or Cdh1. Radiolabeled IVT substrates were added as indicated and incubated for a further 10 minutes. Calcium was added to trigger exit from meiosis-I metaphase arrest and entry into the first cell cycle. Samples were taken at the indicated times and analysed by PAGE and autoradiography. (C) Radiolabeled IVT products were prepared, using 40  $\mu\text{g } \mu\text{l}^{-1}$  DNA template or 20  $\mu\text{g } \mu\text{l}^{-1}$  IVT and purified mRNA template. Templates were: (1) *Xenopus* Aur-A DNA; (2) human Aur-A DNA; (3) *Xenopus* Aur-A mRNA; (4) human Aur-A mRNA; (5) human Aur-A D-box-mutant DNA; (6) human Aur-A (66-403) DNA; and (7) human Aur-A DNA in the presence of 10  $\text{ng } \mu\text{l}^{-1}$  Cdh1(1-125) protein. (D) Following coupled in vitro transcription-translation of the indicated templates for 90 minutes in rabbit reticulocyte lysate, 100  $\mu\text{g } \mu\text{l}^{-1}$  cycloheximide (CHX) was added to inhibit further protein synthesis. Reaction mixes were shifted to 23°C and samples were taken at subsequent times for analysis by SDS-PAGE followed by autoradiography. Where indicated, the proteasome inhibitor MG-115 (1.6  $\mu\text{M}$ ) was added 10 minutes before CHX.

analysis of the sequences required for Cdh1-induced destruction of human Aur-A. For this work, we chose to use *Xenopus* egg extract, because it is much easier to obtain in large volumes than somatic cell G1 extract. However, the first results were completely unexpected. Unlike *Xenopus* Aur-A IVT product, human Aur-A IVT product was destroyed in egg extracts during meiotic exit, even without the addition of Cdh1 (Fig. 3A). This 'Cdh1-independent destruction' nevertheless required the D box, A box and proteasome activity (Fig. 3B).

We had previously noticed that the yields of radiolabeled rabbit reticulocyte IVT products encoded by human Aur-A were significantly lower than those of *Xenopus* Aur-A (Fig. 3C). This difference was encountered both with coupled transcription-translation reactions and IVT of purified mRNA. We initially attributed the low yield of human Aur-A IVT product to poor translational efficiency. However, the observation that mutation of either the D box or the A box resulted in higher yields of human Aur-A IVT products (Fig. 3C) suggested that D-box- and A-box-dependent destruction of human Aur-A was also occurring in reticulocyte lysate. The results of cycloheximide chase experiments confirmed this: *Xenopus* Aur-A IVT product was stable in reticulocyte lysate, but human Aur-A IVT product was unstable (Fig. 3D). Mutation of the D box, truncation of the first 65 residues containing the A box or prior addition of the proteasome inhibitor MG-115 each blocked turnover of human Aur-A IVT in reticulocyte lysate (Fig. 3D). Thus, human Aur-A is sensitive to mitotic-destruction-signal-dependent proteolysis in rabbit reticulocyte lysate.

Because earlier assays with G1-phase HeLa-cell extracts had used IVT products as substrates (Fig. 1), it was important to know whether the destruction seen in those assays was attributable solely to components present in the reticulocyte lysate or was in fact dependent on activities provided by the HeLa-cell G1 extracts. IVT products are routinely frozen in aliquots in preparation for destruction assays. After one freeze-thaw cycle, human Aur-A IVT product was still markedly unstable in reticulocyte lysate, indicating that the destruction activity largely survived freezing (Fig. 3D). However, upon dilution of the thawed IVT product into the volume of cell extract buffer used in the destruction assay, the IVT Aur-A was stable (Fig. 3D, bottom right). Thus, it seems unlikely that components present in the reticulocyte lysate were solely responsible for the destruction of human Aur-A IVT product that was observed in G1-phase HeLa-cell extracts.

One obvious explanation for the D-box- and A-box-dependent turnover of human Aur-A in rabbit reticulocyte lysate is that reticulocyte lysate contains both APC/C and Cdh1, and that these recognize mammalian target proteins more efficiently than frog target proteins. We spent considerable efforts trying to detect Cdh1 and the APC/C subunit Cdc27 in reticulocyte lysate but, unfortunately, none of the available antibodies was informative. Nevertheless, the idea is supported by two pieces of experimental evidence. First, we asked whether the previously characterized dominant negative version of human Cdh1, Cdh1(1-125) (Fig. 2A) (Pfleger et al., 2001), could interfere with the destruction of human Aur-A in reticulocyte lysate. As shown in Fig. 4A, 10 ng  $\mu\text{l}^{-1}$  Cdh1(1-125) protein significantly slowed the turnover of human Aur-A. Second, although *Xenopus* Aur-A IVT is not subject to rapid turnover in reticulocyte lysate (Fig. 3D), we thought that

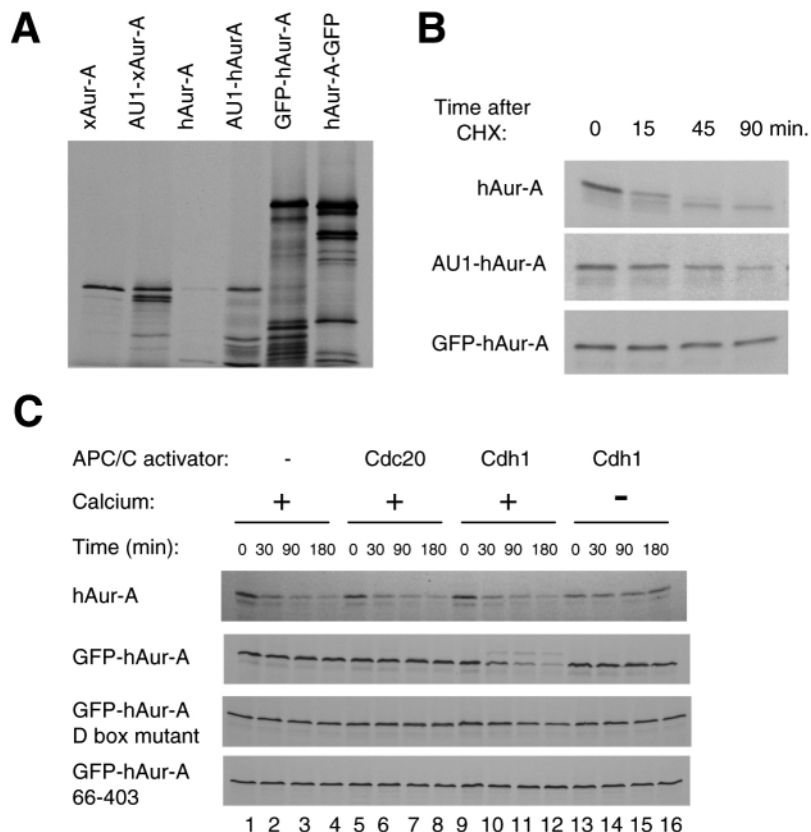


**Fig. 4.** Reticulocyte lysate contains a Cdh1-dependent destruction activity. (A) Destruction of Aur-A in reticulocyte lysate was analysed as follows. Following coupled in vitro transcription-translation of the indicated templates for 90 minutes in rabbit reticulocyte lysate, 100  $\mu\text{g ml}^{-1}$  cycloheximide (CHX) was added to inhibit further protein synthesis. Reaction mixes were shifted to 23°C and samples were taken at subsequent times for analysis by SDS-PAGE followed by autoradiography. Five minutes prior to cycloheximide (CHX) addition, 10 ng  $\mu\text{l}^{-1}$  Cdh1(1-125) protein was added as indicated. (B) *Xenopus* egg CSF extract was incubated for 5 minutes with Cdh1 protein, reticulocyte lysate (RL), or reticulocyte lysate and Cdh1(1-125) protein. Calcium was added to trigger exit from meiosis I. Samples were taken at the indicated times and analysed by PAGE and western blot for endogenous *Xenopus* Aur-A.

addition of large amounts of reticulocyte lysate to egg extract might supply sufficient Cdh1 to permit some destruction of the endogenous *Xenopus* Aur-A during meiotic exit. As shown in Fig. 4B, addition of 5  $\mu\text{l}$  reticulocyte lysate per 1  $\mu\text{l}$  egg extract (instead of the 50 nl normally used in destruction assays) was indeed sufficient to induce detectable destruction of the endogenous *Xenopus* Aur-A during meiotic exit. Importantly, the presence of 10 ng  $\mu\text{l}^{-1}$  Cdh1(1-125) largely blocked this destruction, suggesting that Cdh1 supplied by the reticulocyte lysate was at least partly responsible. Together, these data support the idea that reticulocyte lysate contains Cdh1 that can contribute to the destruction of APC/C substrates under certain experimental circumstances.

#### Epitope tags can reduce the efficiency of Cdh1-dependent destruction

The *Xenopus* Aur-A IVT product used in Fig. 3C and our previous work (Littlepage and Ruderman, 2002) contained a small N-terminal epitope tag (AU1, amino acid sequence DTYRYI), whereas the human Aur-A IVT product used here did not (Fig. 3C). We thus investigated how that tag might influence the destruction of Aur-A in vitro. As shown in Fig. 5A, the yield of *Xenopus* Aur-A was not affected by the presence or absence of an AU1 tag; by contrast, addition of an AU1 tag dramatically improved the yield of human Aur-A IVT product. This increase appears to be at least partly due to a reduced rate of proteolysis, as indicated by cycloheximide

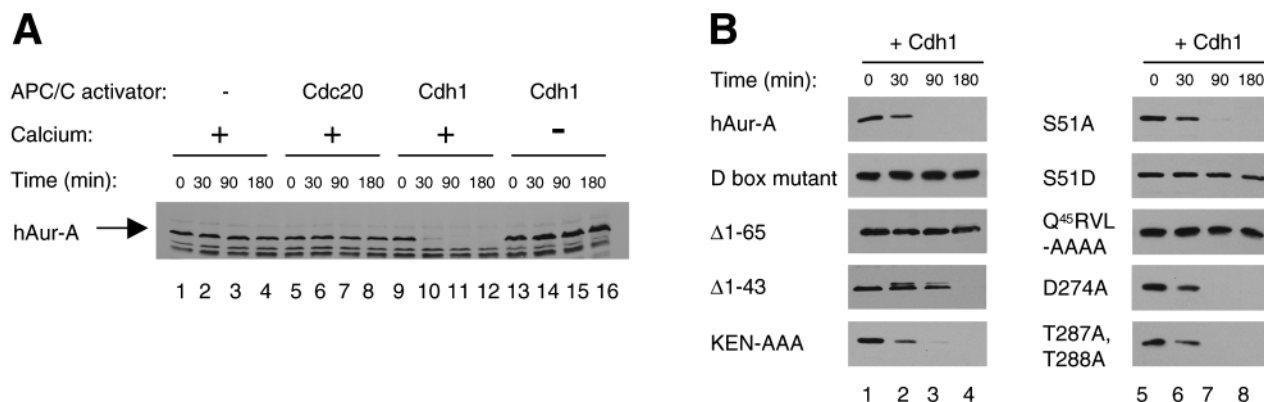


**Fig. 5.** Epitope tags reduce the destruction of Aur-A in vitro. (A) Radiolabeled IVT products were prepared for the indicated proteins using 0.2  $\mu$ g DNA templates: (1) *Xenopus* Aur-A; (2) N-terminal AU1-tagged *Xenopus* Aur-A; (3) human Aur-A; (4) N-terminal AU1-tagged human Aur-A; (5) N-terminal GFP-tagged human Aur-A; and (6) C-terminal GFP-tagged human Aur-A. (B) IVT products were incubated with G1-phase HeLa-cell lysates. Samples were taken at the indicated times and monitored by SDS-PAGE followed by autoradiography. (C) Destruction assays were performed in *Xenopus* egg extracts as described in Fig. 3A.

chase experiments (Fig. 5B). We next tested the effect of a much larger tag, GFP. The GFP tag led to very high yields of human Aur-A IVT product (Fig. 5A) and almost completely stabilized human Aur-A in rabbit reticulocyte lysate (Fig. 5B). Strikingly, the GFP tag also restored the requirement for additional Cdh1 to destroy IVT human Aur-A in *Xenopus* egg extract (Fig. 5C). These observations together indicate that the presence of an epitope tag can sometimes significantly reduce the efficiency of destruction of an APC/C substrate in vitro. This finding has obvious potential relevance for studies that follow the kinetics of destruction of GFP-tagged APC/C substrates in living cells.

#### Destruction of purified recombinant human Aur-A protein in *Xenopus* egg extracts requires Cdh1 and a QRVL tetrapeptide within the A box

Our results so far suggest that rabbit reticulocyte lysate contains Cdh1-dependent destruction activity that recognizes both the D box and the A box. This activity clearly has the potential to confound interpretations of studies carried out using rabbit reticulocyte lysate IVT as the source of radiolabeled test substrates, whether in frog egg or human somatic cell extracts. In view of these newly recognized caveats, it was important to test the ability of purified recombinant human Aur-A protein to undergo destruction in vitro. Wild-type human Aur-A protein was expressed in Sf9 insect cells, purified and then added to frog egg extracts. As shown in Fig. 6A, it was stable in the absence of calcium and remained stable following the addition of calcium. Like endogenous frog Aur-A, the addition of Cdh1 permitted calcium-induced destruction of human



**Fig. 6.** Destruction of purified, recombinant human Aur-A protein in *Xenopus* egg extract requires the D box and sequences within the A box. Destruction assays were performed in *Xenopus* egg extracts as follows. *Xenopus* egg CSF extract was incubated for 5 minutes with the APC/C activators Cdc20 or Cdh1. His<sub>6</sub>-tagged human Aur-A wild-type (A) or mutant (B) protein substrates were added at a final concentration of 220 nM and incubated for a further 10 minutes. Calcium was added to trigger exit from meiosis-II metaphase arrest and entry into the first cell cycle. Samples were taken at the indicated times and analysed SDS-PAGE and western blotted with anti-Aur-A antibodies.

Aur-A protein during M-phase exit (Fig. 6A, lanes 9-12), whereas addition of Cdc20 did not (Fig. 6A, lanes 5-8). Thus, like *Xenopus* Aur-A, destruction of human Aur-A begins during M-phase exit in *Xenopus* egg extracts and is Cdh1 dependent. Gratifyingly, mutation of the D-box sequence (R<sup>371</sup>PML to APMA), truncation of the N-terminal 65 amino acids (containing the A box) and the S53D mutation within the A box each blocked destruction in this assay, whereas the S51A mutation and mutation of the KEN box did not (Fig. 6B). Thus, the same sequences are required to destroy purified recombinant human Aur-A protein during exit from meiosis in egg extract as were defined for IVT human Aur-A in G1-phase human cell extract (Fig. 1).

We used this assay to test the requirement for a very obvious sequence within the A box that has not been previously examined. As shown in Fig. 6B, removal of the first 43 residues (44-403) delayed but did not abolish destruction. By contrast, mutation of the highly conserved tetrapeptide motif Q<sup>45</sup>RVL within the A box completely blocked destruction. The other recombinant proteins tested all behaved exactly as predicted from our previous work and that of others (Fig. 1) (Littlepage and Ruderman, 2002; Castro et al., 2002). Mutations of the D box and the S51D mutation blocked destruction of human Aur-A, whereas kinase-inactivating mutations (D274A and T287A/T288A) and S51A did not. Thus, the use of recombinant protein substrates is strongly recommended for in vitro destruction assays whenever possible.

## Discussion

The work described here establishes the following new points. First, the sequence requirements for the mitotic destruction of human Aur-A in the somatic cell cycle are identical to those previously defined using *Xenopus* Aur-A. These sequences include the C-terminal D box, the A box, the highly conserved QRVL sequence within the A box and serine 51, a conserved residue with the A box whose phosphorylation might protect Aur-A from destruction until mitotic exit. Second, the presence of epitope tags, including GFP, can significantly stabilize Aur-A, at least in vitro. This finding has potential significance for investigations that use GFP-tagged Aur-A to follow the regulation, timing and kinetics of Aur-A destruction in live cell imaging. Third, reticulocyte lysate contains one or more D-box- and A-box-dependent destruction activities that, in the absence of any other components, can degrade human Aur-A IVT product in a Cdh1-dependent manner.

The results of previous investigations were consistent with the idea that Aur-A is stable during the early stages of mitosis and begins to be degraded sometime during mitotic exit. Relevant findings included: (1) the presence of two APC/C-Cdh1-recognition signals, the KEN box and the D box in Aur-A (Arlot-Bonnemains et al., 2001; Littlepage and Ruderman, 2002; Castro et al., 2002; Taguchi et al., 2002); (2) a strong reduction in the amounts of Aur-A protein in post-mitotic cells (Bischoff et al., 1998; Lindon and Pines, 2004); (3) the fact that Aur-A is destroyed in lysates of G1-phase cells (Arlot-Bonnemains et al., 2001); and (4) the ability of Cdh1 overexpression to decrease levels of Aur-A, both in vitro and in vivo (Littlepage and Ruderman, 2002; Castro et al., 2002; Taguchi et al., 2002). Experiments in which proteasome inhibitors were found to block destruction of Aur-A (Honda et

al., 2000; Taguchi et al., 2002; Walter et al., 2000) were consistent with regulated destruction but could not distinguish between direct inhibition of Aur-A destruction and indirect inhibition by blocking cell-cycle progression at an earlier stage. In our work, direct comparisons of the stability of Aur-A protein in lysates of M-phase and early G1 phase cells, which are free from complicating issues of changes in synthesis rates, clearly show that destruction of Aur-A is in fact turned on during mitotic exit.

The finding that rabbit reticulocyte lysate contains A-box-, D-box- and Cdh1-dependent destruction activity has direct relevance for experiments that use radiolabeled IVT products as substrates. This approach has been used widely and successfully in screens to find new APC/C substrates where, for example, they are identified by their selective disappearance after incubation with mitotic lysates (McGarry and Kirschner, 1998; Zou et al., 1999; Funabiki and Murray, 2000; Ayad et al., 2003). Given that human Aur-A shows 'mitotic' destruction characteristics in reticulocyte lysate even before incubation with M-phase lysate, that approach clearly has the potential to miss other important APC/C substrates. We do not know why human Aur-A is more sensitive to destruction by reticulocyte lysate than *Xenopus* Aur-A. One obvious possibility is that the one or more of the relevant components present in these lysates prepared from rabbit (mammal) reticulocytes interact better with mammalian orthologs. This idea would explain the low IVT yields of human Aur-A, as shown here, and human cyclin B1 (R.C., unpublished) relative to their *Xenopus* counterparts.

A second caution comes from the finding that the presence of two different epitope tags, GFP and AU1, each reduced the kinetics and extent of Aur-A destruction. Thus, GFP-tagged Aur-A, and possibly certain other GFP-tagged APC/C substrates that are used for live cell imaging, might not be as efficiently recognized by APC/C as their endogenous counterparts. GFP tags on the N-terminus of Aur-A might also reduce interactions with proteins that are required for the localization of Aur-A to centrosomes (Roghi et al., 1998) and for inhibition of Aur-A activity by the tumor suppressor p53 (Chen et al., 2002).

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